INDUCTION OF PROTEIN SYNTHESIS IN MITOCHONDRIA BY EXOGENOUS RNA. SYNTHESIS OF RABBIT GLOBIN BY ISOLATED MITOCHONDRIA OF *TETRAHYMENA PYRIFORMIS*

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1. Introduction

Cenetic as well as biochemical evidence, has by now established that the information for the synthesis of some mitochondrial proteins originates within the mitochondria, while for others within the nucleus. What is not at all clear is the site of translation of the nuclear messages. One possibility is that the messages are translated within the cytoplasm, and the polypeptides are then, somehow, carried into the mitochondria. Evidence along this line has mainly been presented by Kellems and Butow [1] who showed that the 80S ribosomes attached onto mitochondria, that were first reported by us [2] and later by O'Brien [3], were able to support cycloheximide-sensitive protein synthesis. What remains to be established is that the proteins synthesized on these particular ribosomes are truly mitochondrial. The second possibility is that the nuclear messages themselves migrate inside the mitochondria where they are translated by the mitochondrial protein synthesizing apparatus. Evidence for this second alternative, has been presented by Swanson, who showed that synthetic polynucleotides may enter mitochondria where they can stimulate amino acid incorporation into acid insoluble products [4], by our own group that showed that nuclear 9S RNA enters mitochondria whence it can be reisolated intact [5] and by Gaitskhoki's group that showed that exogenous RNA entering the mitochondria is found associated with ribonucleoprotein particles, that they tentatively identified as mitochondrial ribosomes and polysomes [6]. The discovery inside mitochondria of eukaryote-type poly-A carrying RNA by Avadhani et al. [7] and Perlman et al. [8] and of RNA provirus particles by Kára and his group [9], further supports the second alternative.

In the present communication we present evidence that exogenous RNA from rabbit reticulocytes, stimulates protein synthesis in isolated mitochondria of *Tetrahymena pyriformis*. The stimulation is time as well as concentration-dependent, and is inhibited by chloramphenicol, but not by cycloheximide. Evidence is presented that the α and β chains of hemoglobin are among the products formed.

2. Materials and methods

Radioactive substrates were obtained from Amersham, Bucks, England.

Stock cultures of *Tetrahymena pyriformis*, strain E, were a generous gift of Dr. V. Kapoulas. Mitochondria were prepared from exponentially growing cultures by the method of Hogeboom [10], modified in that the sucrose was dissolved in buffer, composed of 0.1 M Tris–HCl, 0.003 M EDTA and 0.003 M mercaptoethanol, at pH 7.4. The purified mitochondria were suspended in 0.25 M sucrose solution. Rabbits were rendered anemic by six daily subcutaneous injections of phenylhydrazine in 0.15 M sodium acetate pH 7.0 (7.5 mg/kg body weight). Tatal RNA was isolated from reticulocytes by phenol extraction [5]. Rabbit globin was isolated from healthy rabbits by the method of Schapira et al. [11].

Amino acid incorporation into proteins was assayed as follows: *Tetrahymena* mitochondria and reticulocyte RNA were preincubated in centrifuge tubes at 37°C for 5 min in a total volume of 0.6 ml 0.25 M sucrose. One half ml of Beattie and Ibrahim's incubation medium,

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minus the amino acid mixture [12], was then added, as well as 5 μCi of ^{14}C-labelled protein hydrolysate (specific activity 57 mCi/mAtom Carbon), in 0.02 ml. The reaction was stopped by adding 0.1 ml 50% trichloroacetic acid at 90°C, and the tubes were placed in a boiling water bath for 15 min. Following centrifugation at 4°C, and three washes with 5% TCA, the precipitate was dissolved with 3 drops concentrated formic acid, which was then driven off by immersing the tubes in a boiling water bath. The residue was dissolved in H_2O and its radioactivity was counted in a dioxane scintillation cocktail by means of a Packard Tri-Carb liquid scintillation counter. A control tube, that contained all the components of the incubation mixture, except that it was incubated in an icebath, was also similarly processed. The radioactivity of the control tube was subtracted from the radioactivity of the test-mixtures.

3. Results

Each of the experiments presented in this paper, was performed at least three times, with essentially the same results. No difference in the results were found whether ^{14}C-labelled protein hydrolysate or ^{14}C-leucine plus unlabelled amino acid mixture as described by Roodyn et al. [13], was employed.

Fig. 1 shows that amino acid incorporation into acid insoluble products of *Tetrahymena* mitochondria increases, with increasing amounts of reticulocyte RNA. Fig. 2 shows that this incorporation is time-dependent in that it increases up to 30 min and then it decreases. Chloramphenicol inhibits the process while cycloheximide does not (fig. 3). Globin synthesis in mitochondria was detected as follows: *Tetrahymena* mitochondria (1.4 mg of protein) and rabbit reticulocyte RNA (0.2 mg) were preincubated in 2.0 ml Eagle's medium for 5 min at 37°C. The mitochondria were isolated by centrifugation, washed once with 0.25 M sucrose in Tris–EDTA–mercaptoethanol buffer, as described for the isolation of mitochondria above, and were suspended in 2.0 ml of a solution, whose composition has been found optimal for mitochondrial protein synthesis by Beattie and Ibrahim [12], except that L-methionine was omitted. Five μCi ^{35}S-L-methionine in 0.015 ml were added and the mixture was incubated with occasional shaking for

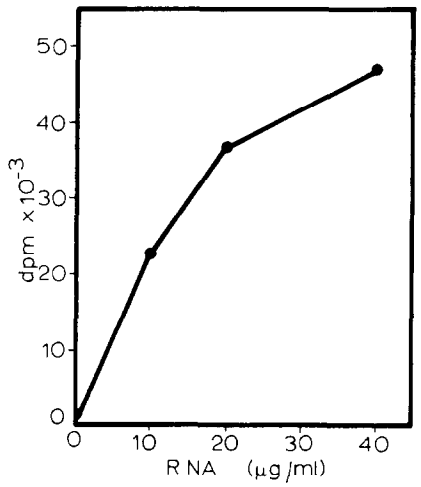

Fig. 1. Effect of increasing amounts of rabbit reticulocyte RNA on the incorporation of ^{14}C-labelled amino acid mixture into hot TCA insoluble products of isolated mitochondria of *Tetrahymena pyriformis*. Incubation for 30 min under the conditions described in the text under Materials and methods. Each sample contained 150 μg/ml mitochondrial protein.

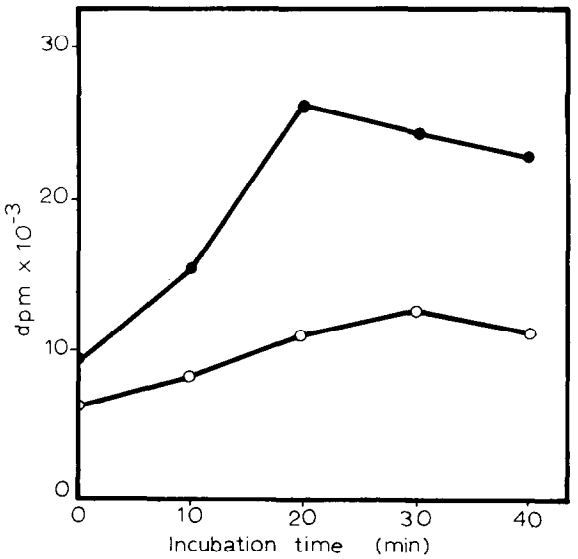

Fig. 2. Time curve of the incorporation of ^{14}C-labelled amino acid mixture into hot TCA insoluble products of isolated mitochondria of *Tetrahymena pyriformis*, , in the presence (●——●) and absence (○——○) of rabbit reticulocyte RNA. Incubation conditions as described in the text under Materials and methods. Each sample contained 90 μg/ml mitochondrial protein and 20 μg/ml reticulocyte RNA.

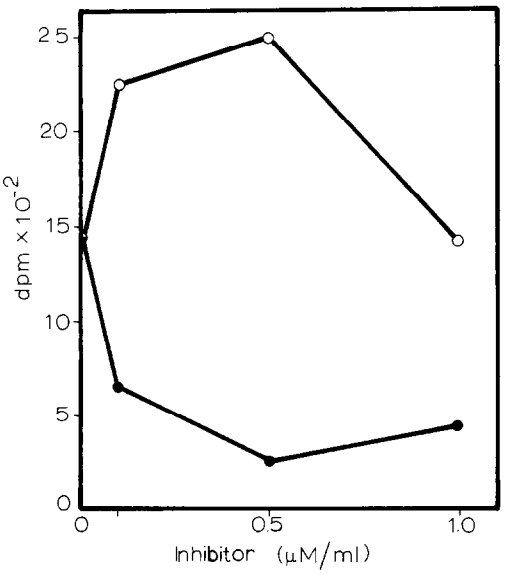

Fig. 3. Effect of varying concentrations of chloramphenicol (●——●) and cycloheximide (○——○), on the incorporation of ^{14}C-labelled amino acid mixture, into hot TCA insoluble products of isolated mitochondria of *Tetrahymena pyriformis*, in the presence of rabbit reticulocyte RNA. Incubation for 30 min under the conditions described in the text under Materials and methods. Each sample contained 50 μg/ml mitochrondrial protein and 12 μg/ml reticulocyte RNA.

25 min at 37°C. The reaction was stopped by immersing the tube in an icebath. Dissolution of the mitochondria was accomplished by rendering the reaction mixture 0.1% with respect to Triton X-100. One half hr later 30 mg of purified rabbit globin, dissolved in 0.02 M pyridin–0.2 N formic acid pH 2.7, were added into the reaction tube, and the mixture was dialyzed against four changes of 1 litre each 0.02 N formic acid buffer pH 2.7, over a 12 hr period.The dialyzed preparation was chromatographed on CM-cellulose (Sigma product 0.73 meq/g), as described by Schapira et al. [11]. Control experiments were performed exactly as above, except that reticulocyte RNA was omitted during preincubation of mitochondria with Eagle's medium.

Column fractions were analyzed for protein according to the method of Lowry et al. [14] and for radioactivity in a Packard Tri-Carb liquid scintillation counter, by adding 1.0 ml of the tube contents into 10 ml of a dioxane scintillation cocktail.

Fig. 4 shows the chromatographic profile for the separation of the α and β chains of rabbit globin. The radioactivity faithfully follows the appearance of protein in all fractions. In the control experiment shown in fig. 5, on the contrary, neither chain is specifically labelled.

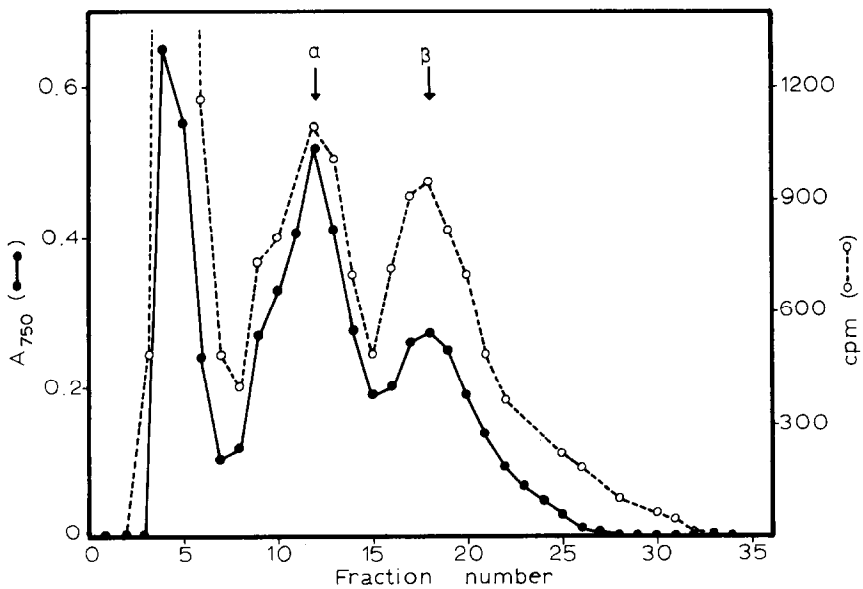

Fig. 4. Chromatography of mitochondrial extract and carrier rabbit globin on CM-cellulose. *Tetrahymena* mitochondria were preincubated in the presence of rabbit reticulocyte RNA and incubated with ^{35}S-methionine as described in the text under Results. ●——● Protein, ○- - -○ radioactivity.

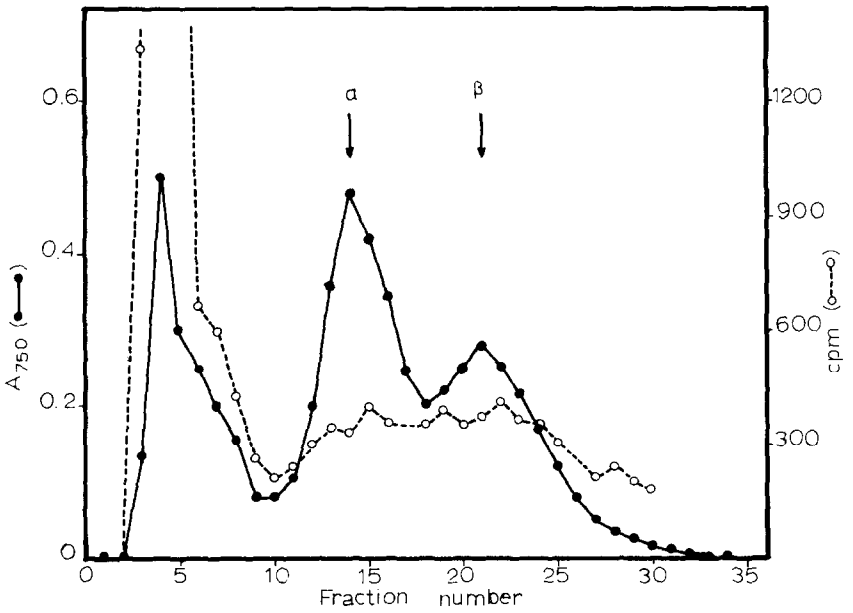

Fig. 5. Chromatography of mitochondrial extract and carrier rabbit globin on CM-cellulose. *Tetrahymena* mitochondria were preincubated in the absence of rabbit reticulocyte RNA and incubated with ^{35}S-methionine as described in the text under Results. •——• Protein, o- - -o radioactivity.

4. Discussion

Our results make it clear that exogenous RNA may increase mitochondrial protein synthesis up to forty-fold depending on the concentration of the RNA used. Proof that we are indeed dealing with mitochondrial-type of protein synthesis is offered by the observation that chloramphenicol, an inhibitor of protein synthesis in prokaryotes and mitochondria, inhibits amino acid incorporation by more than 80%, while cycloheximide, an inhibitor of cytoplasmic protein synthesis in eukaryotes, does not.

The isolation of globin chains from *Tetrahymena* mitochondria, only when reticulocyte RNA is included in the system, constitutes satisfactory proof that the message that directs the synthesis of globin was in fact translated by the mitochondrial protein synthesizing apparatus. We purposely chose to incubate our mitochondria with RNA isolated from a phylogenetically distant species, in order to eliminate probable effects of homologous nuclear RNA, that might have been carried over, during the isolation procedure of the mitochondria. As to the information-al content of rabbit reticulocyte RNA others before us, have shown, that it contains the mRNA for the globin chains [15,16].

Acknowledgement

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